Immunocytochemistry Followed by FISH (Version 2)

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***We present multiple versions of the Immunocytochemistry / FISH protocols because the conditions under which each antibody and DNA probe work can be different and must be determined emperically.**

Reagents

Antifade (1,4-phenylene-diamine)
Bovine Serum Albumin (BSA)
Boehringer Mannheim Biochemicals (BMB), Cat. 100 350
DAPI
BMB, Cat. 236 276
Dextran Sulfate (50%)
Intergen, S4030
Dimethyl sulfoxide (DMSO)
Ethylene glycol bis(succinimidyl succinate)
Sigma, Cat. E3257
Formamide
FLUKA BioChemica, Cat. 47670
Formamide, deionized
Ambion, Cat. 9342
Goat anti-mouse-FITC (FISH 2˚ Ab)
BMB, Cat. 605 240
Goat anti-rabbit-TRITC (ICC 2˚ Ab)
Sigma, Cat. T-5268
Normal Goat Serum
Sigma, Cat. G6767
HCl, 1N
Human Cot-1 DNA
Invitrogen Corp., Cat. 15279-01
Methanol
Mouse anti-biotin-FITC (FISH 1˚ Ab)
Sigma, Cat. F4024
Cot-I DNA (Mouse)
Invitrogen Corp., Cat. 18440-016
Para-Formaldehyde
Sigma, Cat. P6148
Phosphate Buffered Saline, pH 7.4

Invitrogen Corp., Cat. 10010-023
Rabbit polyclonal antibodies (ICC 1˚ Ab)
Specific for desired protein
RNase A
BMB, Cat. 109 169
Salmon testes DNA
Sigma, Cat. D-7657
NaOH, 0.1 M
20X SSC
Tween 20
Sigma, Cat. P1379

Preparation

Methanol
Pre-chill to -20°C

Blocking Solution I (5% NGS/1% BSA/1X PBS)

NGS	500 µl
1%BSA/1X PBS	10 ml

Store at 4°C

Antibody Solution I (1% NGS/1% BSA/1X PBS)

NGS	10 µl
1%BSA/1X PBS	1 ml

Ethylene glycol bis(succinimidyl succinate) (EGS) Solution
Weigh volume of EGS powder [i.e.,100 µl powder] in eppendorf tube
Add equal volume of DMSO [i.e. 100 µl DMSO]
Incubate at 37°C until dissolved and re-determine volume
Calculate concentration based on weight of EGS used, molecular weight of EGS, and final volume of solution (should be ~ 500-650 mM)
Store at RT <1 month
Dilute stock into 1X PBS immediately prior to use for final conc. 50 mM, discard unused portion

1% p-formaldehyde

p-formaldehyde	1 g	
1X PBS	100 ml	
0.1N NaOH, pH 7.4 w/ HCl	500 µl	f.c. [0.5 mM]

*Store <1 month at 4°C

RNase A (DNase-free)
20 mg/ml in sterile water
Boil 15 min, cool to RT, aliquot and store at -20˚C

Master Mix

Dextran sulfate, 50%	40 ml	f.c. [20%]
20X SSC, pH 7.0	20 ml	f.c. [4x SSC]
Sterile dH_2O	40 ml	
Total	**100 ml**	

Vortex solution and place tube on a shaking platform overnight to insure proper mixing.
*Aliquot, and store at -20˚C.

50% FA/SSC

20X SSC	20 ml
dH_2O	80 ml
Formamide	100 ml
Total	**200 ml**

*Adjust pH to 7-7.5 with 1M HCl
Pre-warm to 45°C

0.1X SSC

20X SSC	2.5 ml
dH_2O	498 ml
Total	**500 ml**

Pre-warm to 60°C

4X SSC/Tween 20

20X SSC	200 ml
dH_2O	799 ml
Tween 20	1 ml
Total	**1000 ml**

Pre-warm to 45°C

Blocking Solution II (3% BSA/4X SSC/Tween20)

BSA	0.3 g
4X SSC/Tween 20	10 ml

Store at 4°C

Antibody Solution II (1% BSA/4X SSC/Tween20)

Blocking Solution II	333 µl
4X SSC/Tween 20	666 µl

DAPI (stock solution)
DAPI 2 mg f.c. [0.2 ng/ml]
dH_2O 10 ml
Aliquot and store at -80ºC

DAPI (staining solution)
DAPI stock solution 40 µl f.c. [80 ng/ml]
2X SSC 100 ml

Antifade (1,4-phenylene-diamine)
See Antifade preparation procedure in CGH Protocols

Procedure

1. Grow adherent cells in chamber slides or cytospin suspension cells onto poly-L-lysine coated slides.

2. Fix cells in methanol (pre-chilled to -20˚C) for 10 min at RT.

3. Wash 3 x 5 min 1X PBS at RT.

4. Block coverslips with 25 µl blocking solution I in hybridization chamber 30 min at 37˚C.

5. Incubate with rabbit polyclonal (ICC 1˚ Ab) in 25 µl antibody solution I in hybridization chamber at 37˚C for 60 min.

6. Wash 3 x 5 min with 1X PBS at RT.

7. Incubate with ICC 2˚ Ab [goat anti-rabbit-TRITC; 1:200 in 25 µl antibody solution I] in hybridization chamber at 37˚C for 60 min.

8. Wash 3 x 5 min with 1X PBS at RT.

9a. Incubate with 25 µl EGS solution [dilute stock to 50mM in 1X PBS prior to use and mix well (will be turbid)] in hybridization chamber at 37˚C for 30 min to allow postfixation cross-linking of the Ab to the target protein.

10a. Wash 3 x 5 min with 1X PBS at RT.

OR

9b. Incubate with 25 µl 1% p-formaldehyde [1g p-formaldehyde, 100 ml 1X PBS, 0.5 mM NaOH, adjust to pH 7.4 with HCl (store <1 month at 4˚C)] at RT for 5 min.

10b. Wash 3 x 5 min with 1X PBS at RT.

Note:
Can counterstain with DAPI at this point and mount slides with antifade to have a look at them and determine if Ab detection worked.

Wash coverslips 3 x 5 min in 2X SSC before continuing with procedure.

11. Incubate with RNaseA (1:200 in 1xPBS) in hybridization chamber 60 min at 37˚C

12. Wash 3 x 5 min with 1X PBS.

13. Denature chromosomal DNA by inverting coverslips (18 mm x 18 mm) onto 25 µl drop of NaOH (pH 13.0 - ~0.1M) for exactly 2 min.

14. Rinse immediately in cold 1X PBS.

15. Hybridize denatured/pre-annealed biotin-labeled probe to coverslip (as per standard FISH Protocol, probe is deatured at 80°C, 5 min, in 50% Deionized Formamide/Master Mix and pre-annealed if necessary at 37˚C in the presence of Cot I DNA for 60-90 min).

16. Seal with rubber cement and incubate in hybridization chamber at 37˚C overnight.

17. Remove rubber cement.

18. Wash coverslips 3 x 5 min in FA/SSC (pre-warmed to 45˚C), shaking.

19. Wash coverslips 3 x 5 min in 0.1X SSC (pre-warmed to 60˚C), shaking.

20. Dip slides in 4X SSC/Tween 20 (pre-warmed to 45˚C); do not let dry.

21. Block with 25 µl blocking solution II in hybridization chamber 30 min at 37˚C.

22. Dip slides in 4X SSC/Tween20; do not let dry.

Note: Centrifuge all fluorescent-conjugated Ab for 3 min at 13,000 rpm.

23. Incubate with FISH 1˚ Ab [mouse anti-biotin-FITC, 1:200 in 25 µl antibody solution II] in hybridization chamber 45 min at 37˚C.

24. Wash coverslips 3 x 5 min in 4X SSC/Tween20 (pre-warmed to 45˚C), shaking.

25. Incubate with FISH 2˚ Ab [goat anti-mouse-FITC, 1:200 in 25 µl antibody solution II] in hybridization chamber 45 min at 37˚C.

26. Wash coverslips 3 x 5 min in 4X SSC/Tween 20 (pre-warmed to 45˚C), shaking.

27. Stain for 2 min with DAPI.

28. Wash in 1X PBS for 10 min, shaking.

29. Mount coverslip with antifade on microscope slide.